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Phagosome Maturation: Steady as She Goes

Toll-like receptors (TLRs) trigger inflammatory signaling in macrophages and enrich on phagosomes, suggesting that TLRs may directly influence phagosome formation and maturation. However, in this issue of *Immunity*, Yates and Russell use carefully defined particles and quantitative methodology to measure phagosome maturation and find no effect of TLR signaling on the process.

Phagocytosis is the process by which cells "eat" a wide variety of particles including microbes, apoptotic cells, necrotic cells, and environmental debris. While often associated with activation of inflammatory responses (as during phagocytosis of microbes), phagocytosis of particles such as apoptotic cells is a normal housekeeping activity and is pointedly not inflammatory. A host of receptors participate in recognition of foreign particles, and these receptors translate recognition into internalization, activation of phagosomal killing mechanisms, acquisition of peptides with which to instruct the acquired immune response, and inflammation. A central question in phagocyte biology is to understand how these demands are balanced to be appropriate to the particle being eaten (Stuart and Ezekowitz, 2005). One family of receptors that recognizes microbes is the Toll-like receptor (TLR) family, which is in large part responsible for the transcriptional inflammatory responses of phagocytes to microorganisms. Although current data indicate that TLRs do not trigger phagocytosis, TLR signaling is, by its very nature, closely associated with phagocytosis. Just as the 1930's era bank robber Willy Sutton was famous for robbing banks because "that's where the money is," many TLRs "go where the microbes are" and are found highly enriched in phagosomes. This has prompted many investigators to hypothesize that it would make sense for TLR signaling on phagosomes to have an effect on phagosome maturation (Underhill and Gantner, 2004).

Once formed, phagosomes steadily acidify and sequentially acquire markers of early endosomes, late endosomes, and lysosomes. Certain pathogens can actively modify phagosome biogenesis, indicating that intracellular signals can regulate the process (Brumell and Grinstein, 2004). Conflicting studies have so far suggested that TLR signaling may speed or slow phagosome maturation. Using macrophages from mice lacking TLR2, TLR4, and the TLR signaling adaptor molecule MyD88, Blander and Medzhitov explored the fate of phagosomes containing gram-negative or gram-positive bacteria (Blander and Medzhitov, 2004). Using standard microscopic methods, they reported that acquisition of lysosomal markers (LAMP-2 protein and the fluorescent LysoTracker dye) was significantly impaired in the absence of TLR signaling. The authors suggested that TLR signaling was necessary to permit fusion of bacteria-containing phagosomes with lysosomes. During phagocytosis of apoptotic cells, however, phagosomes acquired lysosomal markers at equivalent rates in wild-type and TLR-deficient macrophages, and TLR stimulation of wild-type cells did not enhance the rate of maturation of the phagosomes. The authors concluded that TLR signaling from within phagosomes during internalization speeds the rate of maturation of the compartment.

In a contrasting study, Shiratsuchi et al. used two lysosomal tracers to microscopically measure phagosome/lysosome fusion in mouse thioglycolate-elicited peritoneal macrophages internalizing a variety of particles including apoptotic cells, antibody-opsonized particles, and latex beads (Shiratsuchi et al., 2004). They reported that the rate of phagosome/lysosome fusion was increased in macrophages lacking TLR4. In this case, TLR4 appeared to slow the rate of phagosome maturation independent of the presence of any obvious TLR4 ligand. The mechanism by which TLR4 might regulate phagosome maturation in the absence of a ligand is not clear.

While these contrasting reports have helped popularize the idea that TLR signaling regulates phagosome maturation, the data do not yet support a consistent conclusion, nor do they establish mechanism. Now, in a new paper in this issue of *Immunity*, Yates and Russell have used carefully defined particles and quantitative methods for unbiased measurement of phagosome/lysosome fusion to explore the matter (Yates and Russell, 2005). Their data demonstrate that TLR signaling does not influence phagosome maturation. For defined particles, the authors used mannose- or IgG-coupled silica particles that were free of any TLR-stimulating activity. TLR stimulation was added by also coating the beads with LPS (a TLR4 agonist) or PAM_3CSK_4 (a TLR2 agonist). Mouse bone marrow-derived macrophages internalized the particles readily, and the phagosomes acidified to pH 5 within 15 min. Inclusion of TLR agonists on the particles did not enhance the rate of acidification. To clarify whether the acidification accurately reflected the rate of phagosome/lysosome fusion, the authors employed an assay based on fluorescence resonance energy transfer (FRET) between a fluorescent lysosomal tracer and a fluorescent particle being internalized. Again, the data demonstrated that phagosome/lysosome fusion occurs at the same rate whether TLRs are activated or not.

While the Yates and Russell study relied on the purity of their particles to establish whether addition of TLR agonists affected the rate of phagosome maturation, other studies have relied on feeding more complex particles to wild-type, $MyD88^{-/-}$, or $TLR^{-/-}$ macrophages. Yates and Russell observed that macrophages deficient in MyD88 had a slight retardation in phagosome maturation. This observation agrees, in part, with the Blander and Medzhitov study, in that it suggests a role for TLR signaling in stimulating phagosome maturation. However, like the Shiratsuchi et al. study, this effect was entirely independent of the presence of LPS to activate TLR4.

One likely explanation for the discrepancies between the studies is that MyD88-deficient macrophages may

be significantly different from their wild-type counterparts. This could be due to stimulation of the wild-type cells with unidentified endogenous TLR agonists, or to stimulation with trace LPS in culture media. In at least one study, microarray analysis of gene expression in resting wild-type and MyD88$^{-/-}$ bone marrow macrophages revealed significant differences that might be anticipated to affect phagocytosis and inflammatory responses to microbes independent of microbe-stimulated TLR activation (Shi et al., 2003). An unfortunate consequence then of relying solely on TLR/MyD88 knockout cells would be that one could make conclusions on the "acute TLR dependence" of a cellular function that is actually sensitive to the preexisting state of the cell. These differences may not be consistent between laboratories due to differing culture conditions.

The present study clearly demonstrates that TLR signaling does not necessarily modify the rate of phagosome maturation. However, it looked only at one type of macrophage, and it is still possible that TLR signaling modifies phagosome maturation rates after activation of the cells or in other types of phagocytes. For example, IFN-γ treatment of macrophages significantly modifies responses to TLR activation, and could well set up a case in which TLR signaling regulates the rate of phagosome maturation. Similarly, the present study examined only resting bone marrow-derived macrophages, and it is possible that other phagocytes such as dendritic cells, resident peritoneal macrophages, or elicited peritoneal macrophages might behave differently. It is also possible that preexposure to TLR agonists would have altered the rate of phagosome maturation, even though TLR activation during the minutes required for phagosome formation and maturation did not. TLR signaling modifies expression of many genes that are involved in membrane traffic and lysosomal function.

Microbial recognition by macrophages occurs through a variety of different receptors that can trigger phagocytosis through different mechanisms. For example, the actin cytoskeletal structures assembled to internalize complement-opsonized particles are very different from those assembled to internalize IgG-opsonized particles (Allen and Aderem, 1996). It is difficult to rule out the possibility that maturation of phagosomes formed during specific types of phagocytosis might be more highly regulated by TLR signaling than others. Yates and Russell examined internalization of IgG-, mannose-, and phosphatidylserine-coated particles as well as formalin-fixed *S. aureus*. This covers a relatively broad range of phagocytic receptors, but certainly not all. Also, there is growing appreciation that maturation of individual phagosome varies somewhat in an apparently random nature (Griffiths, 2004; Henry et al., 2004). For example, some phagosomes are coated with phosphatidylinositol (3,4,5)-triphosphate for just minutes, while others retain the lipid signaling molecule for at least an hour. Such heterogeneity may be inherent "noise" in a highly redundant system where there are many effective maturation paths. Alternately, there may be several different functional types of phagosomes. For example, while some phagosomes may be simply charged with obliterating internalized particles, others may be stocked with different sets of proteases to maximize diversity in peptide generation. Also, different phagosomal maturation paths may make greater energy demands on the cell, which must be balanced against other functions such as cytokine production and motility. It is possible that TLR signaling on phagosomes could affect the balance of formation of certain classes of phagosomes not apparent in the current Yates and Russell study.

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Selected Reading

Allen, L.A., and Aderem, A. (1996). J. Exp. Med. *184*, 627–637.

Blander, J.M., and Medzhitov, R. (2004). Science *304*, 1014–1018.

Brumell, J.H., and Grinstein, S. (2004). Curr. Opin. Microbiol. *7*, 78–84.

Griffiths, G. (2004). Trends Cell Biol. *14*, 343–351.

Henry, R.M., Hoppe, A.D., Joshi, N., and Swanson, J.A. (2004). J. Cell Biol. *164*, 185–194.

Shi, S., Nathan, C., Schnappinger, D., Drenkow, J., Fuortes, M., Block, E., Ding, A., Gingeras, T.R., Schoolnik, G., Akira, S., et al. (2003). J. Exp. Med. *198*, 987–997.

Shiratsuchi, A., Watanabe, I., Takeuchi, O., Akira, S., and Nakanishi, Y. (2004). J. Immunol. *172*, 2039–2047.

Stuart, L.M., and Ezekowitz, R.A. (2005). Immunity *22*, 539–550.

Underhill, D.M., and Gantner, B. (2004). Microbes Infect. *6*, 1368–1373.

Yates, R.M., and Russell, D.G. (2005). Immunity *23*, this issue, 409–417.